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A RADIOMETRIC HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR PHYSOSTIGMINE

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This method was developed to determine the pharmacokinetic parameters of ^{3}H-physostigmine (^{3}H-Phy) from serial plasma samples of small animals. A 200 ul plasma sample, mixed with 50 uls of aqueous neostigmine (10 mg/ml) to inhibit in vitro metabolism, was membrane filtered (10,000 mol wt limit). Filtrate (150 uls) was injected onto an HPLC instrument containing a 100 ul sample loop, C18 column and flow-through scintillation counter. Flow rates of mobile phase (5.0 mM 1-octanesulfonic acid, 5.0 mM sodium phosphate, 0.175 mM acetic acid aqueous buffer:methanol (60:40)) and scintillation fluid were 1.2 and 4.0 ml/min, respectively. Areas under the curves of cpm versus time for 0.1, 0.5, 1.0 and 5.0 mg ^{3}H-Phy/ml were used to create weighted standard curves by reciprocal of variance (n = 6). Physostigmine was totally resolved from eseroline. Sensitivity for physostigmine was 0.05 ng/ml; extraction efficiency ± SD was 99.6 ± 4.4% (n-

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= 6). Correlation coefficients representing standard curve linearity ranged from 0.9697 to 0.9999 (n = 6). Within-day and between-day coefficients of variation (n = 6) for 0.2, 0.75, 1.5 and 2.5 ng ^{3}H-Phy/ml ranged form 0.7 to 20% and 16 to 32%, respectively. The sensitivity, linearity and precision of this method suggest that it should be able to accurately measure ^{3}H-Phy concentrations in small plasma volumes to obtain pharmacokinetic data for small animals.

PREFACE

The work described in this report was authorized under USAMRICD animal use protocol number 1-02-87-000-B-444, entitled "Methods development for quantifying physostigmine in biological fluids and tissues by HPLC and scintillation counting." The work was started on 12 Jun 1987 and completed on 28 Sep 1987. The experimental data are recorded in USAMRICD notebook number 052-87.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

Physostigmine, originally derived from the calabar bean, is the oldest known carbamate. It reversibly inhibits acetylcholinesterase and has been clinically used to treat patients overdosed with drugs possessing cholinergic activity. Physostigmine has also been shown to be a valuable treatment for Alzheimer's patients (Smith and Swash, 1979).

Small animal studies have revealed the effectiveness of physostigmine as a pretreatment against organophosphate poisoning (Harris et al, 1984). Pharmacokinetic studies are of interest to futher define the dose-response relationship and to extrapolate results to man. However, until recently, pharmacokinetic analyses of physostigmine have been limited by lack of an appropriate analytical technique. Effective doses of physostigmine produce very low concentrations in plasma, requiring extremely sensitive analytical methods.

An HPLC technique has been used to study the pharmacokinetics of ^{3}H-physostigmine in rats (Somani and Khalique, 1985). This technique, however, requires such a large blood volume for each sample that it is unsuitable for serial sampling in small animals. In addition, it is excessively time consuming.

In this paper we present a method to study physostigmine pharmacokinetics in small animals that are serially sampled for blood. The concentration-time profile can be determined in each animal, minimizing variability and animal requirements. The method uses flow-through scintillation counting, thus providing decreased labor and increased speed.

MATERIALS AND METHODS

Materials:

Physostigmine (benzene ring - ^3H) with 16.1 Ci/mmole specific activity was purchased from Amersham International (Arlington Heights, IL). Physostigmine (free base) and neostigmine bromide were obtained from Aldrich Chemical Co. (Milwaukee, WI) and Hoffman-LaRoche Inc. (Nutley, NJ), respectively. Eseroline was obtained from Walter Reed Army Institute of Research, under contract # DAMD17-83-C-3207.

Reagents:

Hydrochloric acid, 1-octanesulfonic acid (sodium salt) and monobasic sodium phosphate were analytical grade. Glacial acetic acid was reagent grade. Methanol, water and Ultrafluor scintillation fluid (National Diagnostics Inc., Somerville, NJ) were HPLC grade. All reagents and chemicals were obtained from commercial sources.

Equipment and HPLC conditions:

A Beckman 341 high pressure liquid chromatography system was equipped with a 100 uL sample loop, a Waters disposable C18 guard column and a stainless steel Waters uBondapack C18 separation column (3.9 mm x 30 cm, 10 micron pore size). Coupled to this was a Radiomatic Instruments Inc. Flo-One B radioactive flow detector with a 2.5 ml sample chamber.

Mobile phase flowed through the column at a rate of 1.5 ml/min. Prior to reaching the detector's sample compartment, the column effluent was mixed with Ultrafluor scintillation fluid in a 4:1 ratio; the resulting mixture achieved a flow rate of 5.5 ml/min through the Flo-One's cell.

The mobile phase was made according to the method of Somani and Khalique (1985). Briefly, an aqueous buffer (0.5 mM 1-octanesulfonic acid, 5 mM monobasic sodium phosphate and 1% acetic acid) was mixed with methanol in a 60:40 (v/v) ratio and the pH was adjusted to 3.1. The mixture was then filtered through a 0.45 micron nylon 66 membrane (Alltech Assoc. Inc.) under vacuum.

Standards:

Plasma spiked with 50 ug neostigmine/ml was used in preparing stock solution of 29.3 mCi ^3H physostigmine/ml plasma. All working solutions were prepared from this stock. Standard ^3H physostigmine concentrations were 0.1, 0.5, 1.0, 2.5 and 5.0 ng/ml.

Sample preparation:

For each analysis, 200 uL of plasma was placed into a Centricon microconcentrator tube (Amicon Corp., Danvers, MA). For unknown samples, 50 uL of a 50 ug/ml solution of neostigmine bromide in distilled water was added to the tubes. Standards already had neostigmine added. The microconcentrator tubes contained a membrane filter with a 10,000 molecular weight cutoff quality. Samples were centrifuged at 7,000 x g and 4 degrees C for 70 minutes in a Dupont Sorvall RC-5B centrifuge. Approximately 150 uL of protein-free filtrate resulted (per sample). The filtrate was kept refrigerated until injection HPLC analysis. Except where noted, all samples were analyzed within several hours of preparation.

Quantitation:

Physostigmine concentrations were determined from weighted standard curves derived each study day from analyses of standard physostigmine concentrations (0.1, 0.5, 1.0, 2.5, and 5.0 ng/ml) in neostigmine spiked plasma. The area under the curve (AUC) values (radioactive counts) for physostigmine from the Flo-One detector were plotted versus the corresponding standard concentrations. Data were analyzed by weighted least squares regression analysis, with weight based upon the reciprocal of the variance of six samples at each concentration. For unknown samples, a multiplication factor (1.25) was applied in the data analysis to adjust for the increased volume resulting from the addition of neostigmine bromide solution to plasma.

Sensitivity:

The sensitivity of the analytical method was defined as the lowest physostigmine concentration producing a peak with an AUC value greater than three times the standard deviation above background.

Precision:

Variability was determined using within-day and between-day coefficients of variation (C.V.'s). For both determinations, data were used from four concentrations (0.2, 0.75, 1.5 and 2.5 ng/ml) of physostigmine in plasma. To estimate within-day variability, three samples of each of the four concentrations were assayed the same day. C.V. values were determined from these three samples for concentrations derived from a daily standard curve. The mean of six sets (one each day) of these C.V. values represented within-day variability.

C.V. values were also calculated from 18 samples of each of the four concentrations assayed over the six days. These values

represented total variability attributed to within-day and between-day differences. The difference of these values from the within-day C.V. values represents day to day (between-day) variability.

Efficiency:

Four samples each of filtered and unfiltered ^{3}H-physostigmine solutions (1 ng/ml mobile phase) were analyzed. The average A.U.C. of filtered solutions was divided by that of the unfiltered to determine efficiency. The standard deviation was computed by the method of estimating the error of a computed result from the errors of component factors (Benedetti-Pichler, 1936).

Protein binding:

The efficiency study was replicated with the filtered ^{3}H-physostigmine-containing plasma replacing the unfiltered mobile phase solution. Concentrations determined from filtered plasma were divided by those from unfiltered mobile phase to determine plasma protein binding.

RESULTS AND DISCUSSION

Because physostigmine is hydrolyzed to eseroline in vitro (Ellis, 1943) and in vivo (Somani and Khalique, 1986), a method must assure resolution of the two. The mobile phase and HPLC column in our study was the same as that of Somani and Khalique (1985) and therefore similar resolution was expected, as depicted in Figure 1. Due to unavailability of radioactive eseroline, the observed separation required ultraviolet detection. HPLC effluent in all other parts of this study went directly to the radioactive flow-through detector. The chromatogram of ^{3}H-physostigmine detected by the flow-through scintillation counter had a slightly longer retention time (10.0 min) than detected by ultraviolet spectrophotometry (8.1 min). The increased time is due to an increase in dead volume from the end of the HPLC column to the detector, and does not significantly affect resolution.

Standard curves were linear ($r^2 = 0.997$) over the entire concentration range (0.1-5.0 ng/ml), as depicted in Figure 2. The detection sensitivity for physostigmine, as assessed by a detected peak above the background by three times the standard deviation, was 0.05 ng/ml.

Inter- and intra-day variabilities, expressed as coefficients of variation, are listed in Table 1. The highest variability (C.V. = 20%) was found within a day at the 0.2 ng/ml concentration. Considering the magnitude of this concentration, a 20% C.V. value is small and indicates a very precise analytical procedure within the concentration range studied.

Efficiency of filtering physostigmine through the microconcentrator tubes was 99.6% (Table 2), indicating no significant adsorption to the filtering tubes. With a 10,000 molecular weight cutoff, the membrane filters provided a clean filtrate to extend HPLC column life and to measure physostigmine binding. The percentage of binding physostigmine to plasma protein was 37%. The majority of physostigmine in plasma is free.

CONCLUSION

A precise and sensitive method has been developed to measure physostigmine concentrations in small volumes of plasma. The method allows for serial sampling, thereby affording better results in generating pharmacokinetic data while using less animals. Future work will apply this method to define pharmacokinetic parameters of physostigmine in small animals.

FIGURE 1

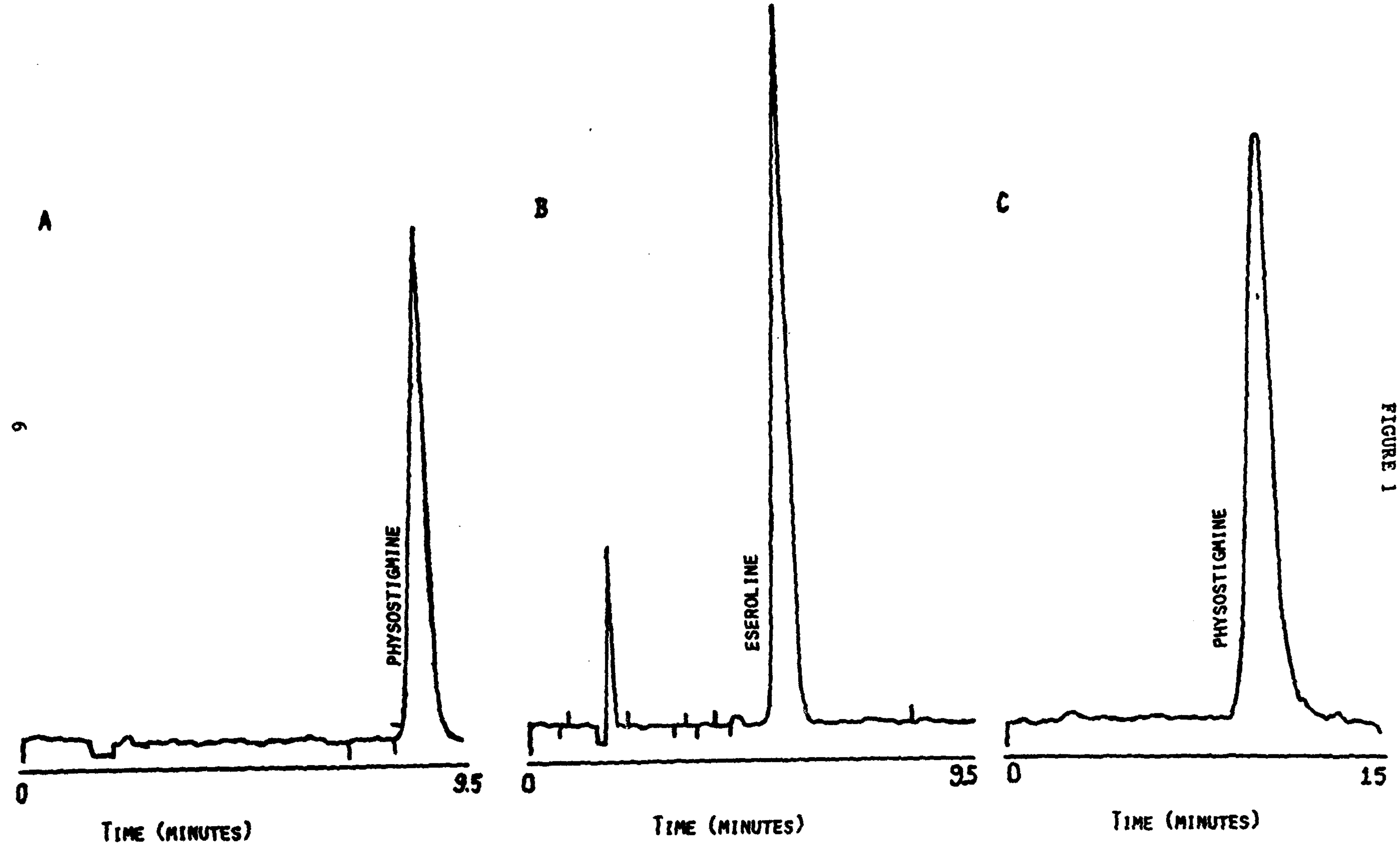

Figure 1. Chromatograms of (A) physostigmine(retention time 8.1 min) and (B) eseroline (retention time 5.8 min) detected by an ultraviolet spectrophotometer and of (C) ^3H-physostigmine (retention time 10.1 min) detected by a flow-through scintillation counter.

FIGURE 2

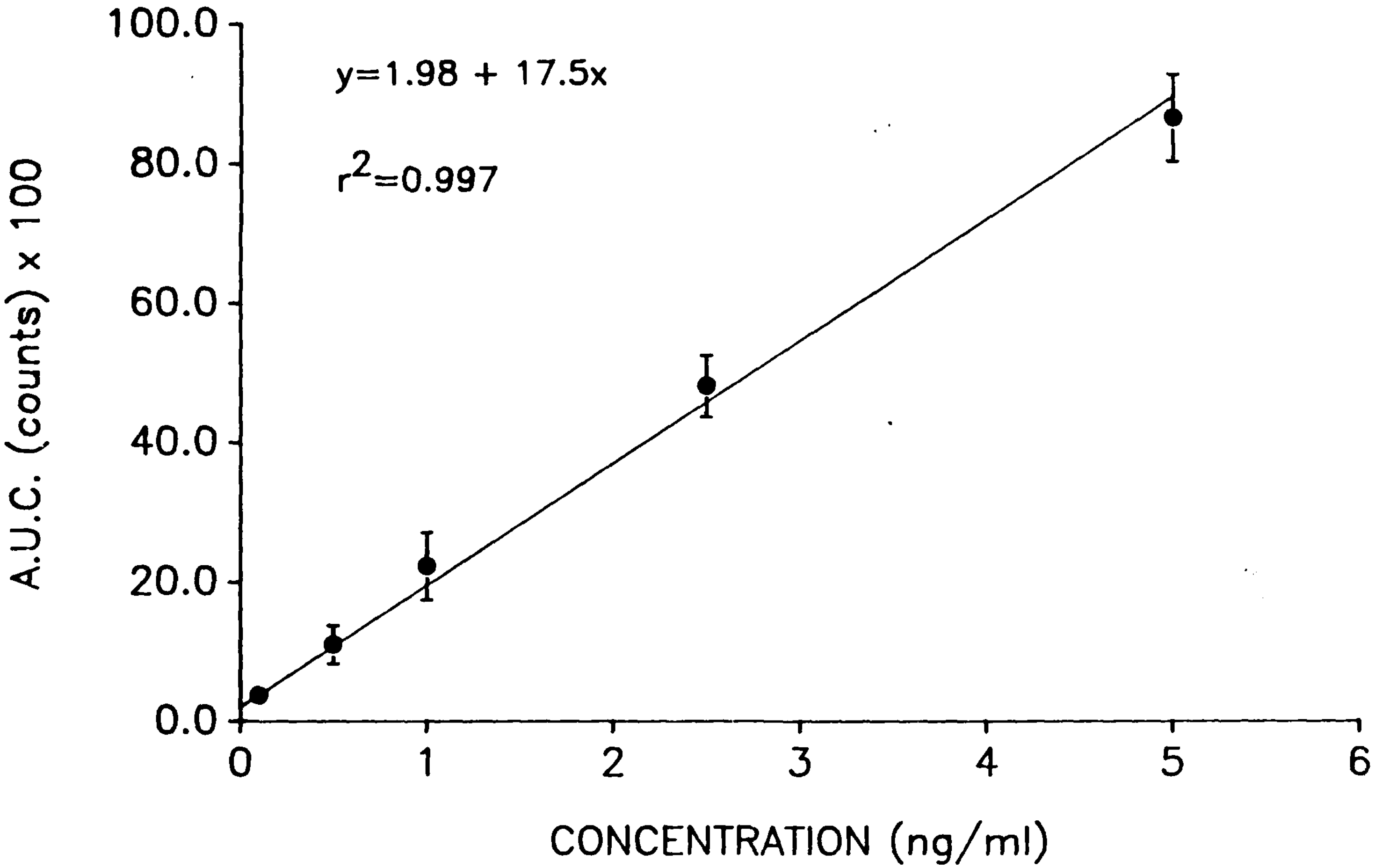

Figure 2. Standard response curve for physostigmine. Five concentrations were analyzed each day, for six days. The means (+SEM) of each concentration were plotted with the best fit line, weighted by the inverse of the variance.

Table I Precision Expressed as Within-day and Between-day Coefficients of Variation (%)

Concentration (ng/ml)	Between-day*	Within-day**
0.2	12.1	20.2
0.75	11.7	4.7
1.5	9.1	7.5
2.5	10.7	9.7

* Eighteen samples of each concentration assayed over six working days were used to calculate among-day C.V. values.

** Means of six within-day C.V. values (each determined from triplicate samples).

Table II Analytical Efficiency and Plasma Protein Binding

Solution*	A.U.C. (radioactive counts)		
	mean*	S. D.	C.V.
Unfiltered mobile phase	4032	111	2.8%
Filtered mobile phase	4014	140	3.5%
Filtered plasma	2550	129	5.0%

Efficiency = 100(filtered/unfiltered mobile phase)
= 99.6%

Protein binding = 100(1 - filtered plasma/filtered mobile phase)
= 36.5%

*--Four samples of each ^{3}H-physostigmine solution (1 ng/ml) were analyzed.

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